

Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection

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Although mucosal surfaces represent the main portal of entry for pathogens, the mechanism of antigen presentation by dendritic cells (DCs) that patrol various mucosal tissues remains unclear. Instead, much effort has focused on the understanding of initiation of immune responses generated against antigens delivered by injection. We examined the contributions of migratory versus lymph node–resident DC populations in antigen presentation to CD4 and CD8 T cells after needle injection, epicutaneous infection, or vaginal mucosal herpes simplex virus (HSV) 1 infection. We show that upon needle injection, HSV-1 became lymph-borne and was rapidly presented by lymph node–resident DCs to CD4 and CD8 T cells. In contrast, after vaginal HSV-1 infection, antigens were largely presented by tissue-derived migrant DCs with delayed kinetics. In addition, migrant DCs made more frequent contact with HSV-specific T cells after vaginal infection compared with epicutaneous infection. Thus, both migrant and resident DCs play an important role in priming CD8 and CD4 T cell responses, and their relative importance depends on the mode of infection *in vivo*.

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Abbreviations used: cDC, conventional DC; DN, double negative; EpCAM, epithelial cell adhesion molecule; f.p., footpad; iNOS, inducible NO synthase; ivag, intravaginal; pDC, plasmacytoid DC; PTX, pertussis toxin; TIP DC, TNF/inducible NO synthase–producing DC.

The internal mucosal surfaces of the human body serve many critical biological functions such as respiration, digestion, and reproduction. Pathogens have devised multiple strategies to invade human hosts through mucosal surfaces. To counter such microbial attacks, the mucosal surfaces are surveyed by specialized DCs capable of mounting effective antiviral immunity in local lymphoid organs (1). Although it was thought that local tissue-resident DCs phagocytose microbial antigens and migrate to the draining lymph node to prime naive T cells (2), this paradigm has been challenged by several recent studies in which lymph node–resident DCs were shown to be the only APCs to present antigens to T cells (3). In particular, in a study using epidermal HSV-1 infection, removal of the infected skin area at early time points resulted in diminished CTL responses (4). However, because no antigen presentation was demonstrated from the migrant DC population and the lymph node–resident CD8 α^+ DCs were exclusively associated with CTL priming capacity, the study implied that the migrant DCs acted as simple ferries for peripheral antigens (5).

With respect to the DCs involved in CTL priming, the importance of the lymph node–resident CD8 α^+ DCs has become well accepted (6). The CD8 α^+ DCs have been shown to present antigens from other cells (7), and from pathogens including HSV-1, influenza virus, vaccinia virus (8), lymphocytic choriomeningitis virus, and *Listeria monocytogenes* (9). The exclusive ability of the CD8 α^+ DCs to prime CTL responses was shown whether the HSV-1 was injected by a needle into the footpad (f.p.) (10), via the intravenous route (8), or by dermal abrasion (11). The cellular basis for the potent ability of the CD8 α^+ DCs to present antigens on MHC class I (MHC I) could not be explained simply by their ability to capture exogenous antigens. By isolating splenic DCs that have captured an equal number of antigen-coated beads after systemic administration of labeled beads into mice, cross-presentation was shown to be much more efficient in CD8 α^+ DCs compared with CD8 α^- DCs (12). Further, CD8 α^+ DCs were shown to express higher levels of proteins

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required for the cross-presentation pathway compared with the CD8 α^- population (13). It is unknown how antigens are acquired by the lymph node–resident CD8 α^+ DCs. It is possible that the CD8 α^+ DCs acquire the antigen from the lymph, as antigens injected into the skin can access the draining lymph nodes within 30 min (14, 15). After f.p. injection of HSV-1, the virus can be found in the draining lymph node from 2–24 h after inoculation (16). Another possibility is that a population of peripheral DCs carry the antigen to the draining lymph node, whereby they transfer the antigen to the resident CD8 α^+ DCs (17), which was demonstrated by infection with HSV-1 after dermal abrasion (4). The lymph node–resident DCs have been shown to acquire antigens from other DCs and present such antigens on MHC class II (MHC II) (18). In addition, migrant DCs collected from influenza-infected mice have been shown to transfer antigen to CD8 α^+ DCs in vitro for presentation to CD8 T cells (19).

If migrant DCs act merely as simple ferries, how are CD8 α^+ DCs licensed to prime CTLs? It is clear that CD8 α^+ DCs mediate the induction of both tolerance and immunity depending on the nature of the encountered antigen (17). For CD4 T cell priming, DCs must be activated through the Toll-like receptor in a cell-autonomous (20) and even in a phagosome-autonomous (21) manner. For CD8 T cell priming after HSV-1 infection, cognate CD4 T cell help is required to license DCs to activate CTLs (22). Therefore, DCs that contact the pathogen and process the microbial information are required to initiate Th1 responses (20, 21), and the interaction of the same DCs with CD4 T cells is necessary for subsequent priming of CTLs (22). These findings suggest that (a) migrant DCs must also carry and transfer microbial information to the lymph node–resident CD8 α^+ DCs, and that (b) CD8 α^+ DCs must also present viral antigens on MHC II to receive help from CD4 T cells. Previous studies have shown that the migrant tissue–derived DCs are responsible for priming CD4 T cells after mucosal HSV-2 infection (23), subcutaneous antigen inoculation (14), or self-antigens (24). More recently, monocyte-derived dermal DCs were shown to migrate to the cutaneous lymph nodes and present parasite antigens, and induced protective Th1 responses against *Leishmania major* (25). In addition, after lentivirus infection, priming of CD8 T cell responses depends on the skin-migrant and not lymph node–resident DCs (26). However, most studies to date examined in vivo DC handling of antigen for MHC I or II presentation but not both simultaneously, failing to provide an integrated picture of antigen presentation by migrant versus resident DCs to CD4 and CD8 T cells in the lymph node.

A vast majority of the studies to date use needle inoculation of antigens or infectious agents to study the mechanism by which immune responses are initiated. However, although the use of needles to inject antigens might very well mimic priming by vaccines or systemic infection, immune-inductive mechanisms defined by needle-injected viruses might not necessarily be applicable to natural infection by the same virus. Importantly, the needle-based inoculations of HSV-1 do not allow infection of the natural targets of viral replication, namely the mucosal epithelial cells. Consequently, the access to viral

antigens and pathogen-associated molecular patterns by local and lymph node DCs could vary depending on the routes of virus administration. For example, it is well known that needle-injected antigens access distinct regions of the lymph node via conduits (15) and are taken up and presented by lymph node DCs (27). However, it is unknown how readily antigens or viruses access the lymph via mucosal application. To address these issues, we examined the population of DCs that present viral antigen to CD4 and CD8 T cells in the draining lymph nodes after needle, epicutaneous versus mucosal infection with HSV-1. Differential contributions of the tissue-derived and lymph node–resident DCs in antigen presentation to T cells were found depending on the route of infection.

RESULTS

Both CD8 α^+ and CD8 α^- DCs, but not plasmacytoid DCs (pDCs), present viral antigens to CD4 and CD8 T cells after mucosal and needle HSV-1 infection with differential kinetics

We examined the ability of the DC subsets in presenting viral antigens to CD8 T cells in vivo. Because CD8 α^+ DCs (10) and CD8 α^- DCs (23) were shown exclusively to prime CTL and Th1 responses after cutaneous and mucosal infection with HSV-1 and -2, respectively, to eliminate any variables associated with the two types of HSVs used, we focused on the priming capacity of DC populations after HSV-1 infection via f.p. or intravaginal (ivag) inoculation in parallel. This allowed critical comparison of the mechanisms involved in immune induction after needle subcutaneous injection and mucosal infection with the same virus.

Three DC subsets were sorted from the draining lymph nodes of f.p.- (popliteal) and ivag-infected (inguinal and iliac) mice at 20 and 72 h after infection, as described in Fig. S1 (available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>). As reported previously (10), f.p. injection of HSV-1 resulted in presentation by CD8 α^+ DCs as early as 20 h after infection (Fig. 1, A and B). In addition, using a revised DC sorting strategy (Fig. S1), we saw robust antigen presentation by the double-negative (DN) DCs to CD8 T cells (Fig. 1, A and B). The DCs involved in antigen presentation to CD8 T cells were similar whether we used naive gBT-I TCR transgenic CD8 T cells (Fig. 1, A, D, G, and J) or bulk CD8 T cells from mice immunized with HSV-1 (Fig. 1, B, E, H, and K) as the responder cells. Similar involvement of the respective DC subsets was observed for CD4 T cell activation (Fig. 1 C). In contrast, no significant priming by any of the DC subsets was observed in the lymph nodes of ivag-infected mice at 20 h after infection (Fig. 1, D–F). By 72 h, antigen presentation was observed in the lymph nodes of mucosally infected mice. Following both routes of infection, CD8 α^+ and CD8 α^- DCs, but not pDCs, were found to present viral peptide to CD8 T cells (Fig. 1, G, H, J, and K). This was not only true for IFN- γ secretion but also with respect to the proliferation induced in gBT-I cells by varying numbers of the sorted DCs used (Fig. S2). Examination of the DC subsets that present viral peptides to CD4 T cells revealed a very similar pattern: CD8 α^+ and DN DCs, but not pDCs, presented viral antigens to CD4 T cells by 72 h (Fig. 1, I and L).

Further examination of the three DC subsets revealed that, on a per cell basis, pDCs were roughly 10 times less efficient in stimulating naive CD8 T cell proliferation (Fig. S3 A) and IFN- γ secretion (Fig. S3 B) *in vitro* after exogenous peptide pulse compared with the other two DC subsets. To determine whether the difference in the kinetics of antigen presentation by the DC subsets depends on the virally infected organ (skin vs. vagina) or on the mode of delivery (needle injection vs. mucosal inoculation), we examined presentation of viral antigen by lymph node DCs after submucosal needle injection of HSV-1 into the vaginal cavity. The kinetics of antigen presentation after needle delivery of the virus into the vagina was similar to the needle injection of the virus into the f.p., in that the antigens were presented at 20 h (Fig. S4), but differed significantly from the vaginal mucosal infection (Fig. 1). These data clearly demonstrated that the kinetics of T cell priming are not dictated by the tissue in which the virus is introduced (skin vs. vagina) but are dictated by the mode of virus inoculation (needle vs. mucosal).

To reconcile the difference between these observations and previously reported studies, we compared the DC populations sorted by our method (Fig. S1) to the one described by others (Fig. 2) (8, 10, 11). DC subsets purified by the latter method resulted in CD8 T cell priming activity mainly restricted to the CD8 α^+ DC population (Fig. 2 C), as shown previously (10). Upon further examination, it was found that the DN DC population sorted accordingly with the previously described method was contaminated with NK cells,

particularly after viral infection (Fig. 2 A). Because NK cells contaminate cells gated solely on the CD11c expression (28), especially after inflammation (29), it is possible that the contaminating NK cells in the DN DC gate could hinder the ability of these cells to present antigen to CD8 T cells. Indeed, removal of the NK cell contamination from the DN DC gated cells resulted in robust activation of CD4 and CD8 T cells by the DN DCs (Fig. 2, B and C). Thus, throughout the rest of the study, we opted to deplete NK cells from our starting population of DCs before flow cytometric cell sorting.

CD8 α^- DCs constitute the majority of the APCs in the lymph node

Having determined the ability of DC subsets to present viral peptide on a per cell basis, we next wished to examine the impact of such presentation by each DC subtype in the context of the entire animal. Because antigen presentation occurs exclusively in the draining lymph nodes after HSV-1 infection (30), we focused on the DC subsets within the draining lymph nodes after f.p. and ivag infection. First, the total number of DCs that accumulate in the draining lymph node was assessed. In the steady state, the total numbers of DC populations in the one popliteal lymph node draining the f.p. are significantly less than those of the vaginal draining lymph nodes (mean of iliac and inguinal nodes) because of differences in the lymph node size. f.p. injection of HSV-1 resulted in the accumulation of all DC subsets starting at 20 h after infection (Fig. 3 A), whereas mucosal

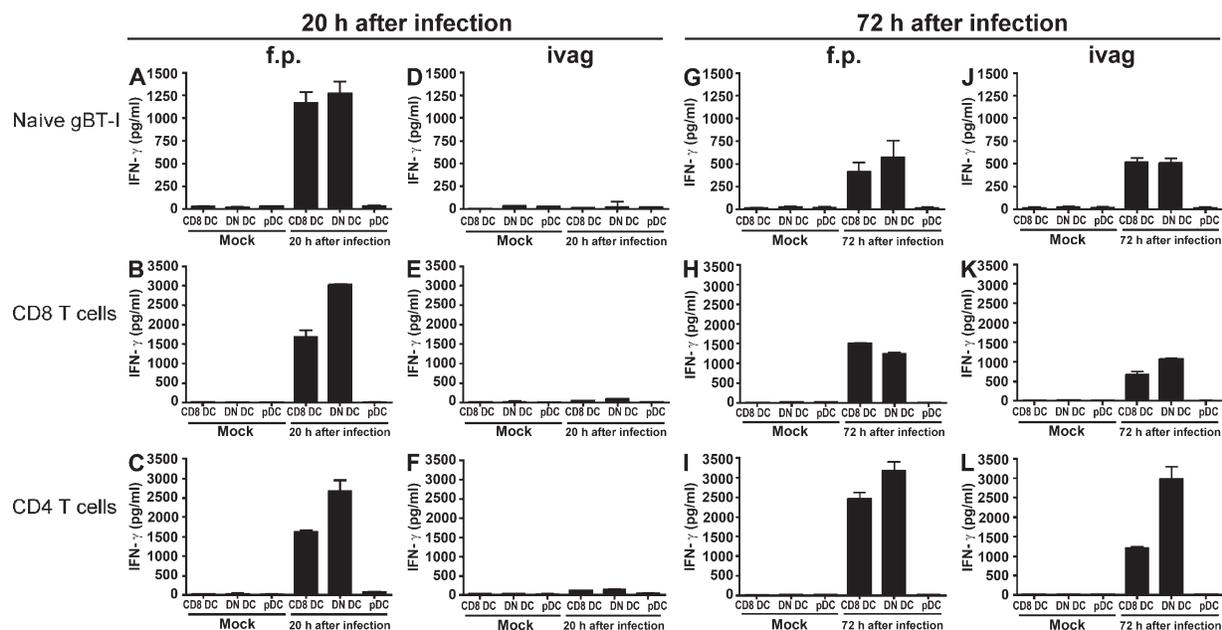


Figure 1. Both CD8 α^+ and CD8 α^- DCs, but not pDCs, present viral antigens to CD4 and CD8 T cells after mucosal and cutaneous HSV-1 infection. Groups of mice were infected with 10^6 PFU HSV-1 via f.p. (A–C and G–I) or ivag (D–F and J–L) and at 20 h (A–F) or 72 h (G–L) after infection, the draining lymph nodes were collected, DCs were enriched, and CD8 α^+ DCs, pDCs, and DN DCs were sorted as described in Fig. S1 (A and B; available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>). 25,000 sorted DCs were cocultured with 10^5 HSV-1-specific naive gBT-I cells (A, D, G, and J), bulk effector CD8 T cells (B, E, H, and K), or bulk effector CD4 T cells (C, F, I, and L) for 72 h, and IFN- γ secretion was measured. These results are representative of three similar experiments. Data are means \pm SD.

HSV-1 infection resulted in the increase in DC subsets around 72 h after infection (Fig. 3 B), consistent with the kinetics of antigen presentation (Fig. 1). Notably, the number of DN DCs always far exceeded the number of CD8 α^+ DCs at all time points. Taking into consideration the total numbers of these DC subsets, their contributions in both CD4 and CD8 T cell priming were calculated on a per lymph node basis. This was accomplished by multiplying the total number of DCs to the ability of a given population of 25,000 sorted DCs to stimulate T cell responses (see Materials and methods). This analysis revealed that, because of the greater number of DN DCs, the majority of T cell priming in the host was mediated by the DN DCs, whereas CD8 α^+ DCs contributed to a lesser extent after f.p. and ivag HSV-1 infections (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>).

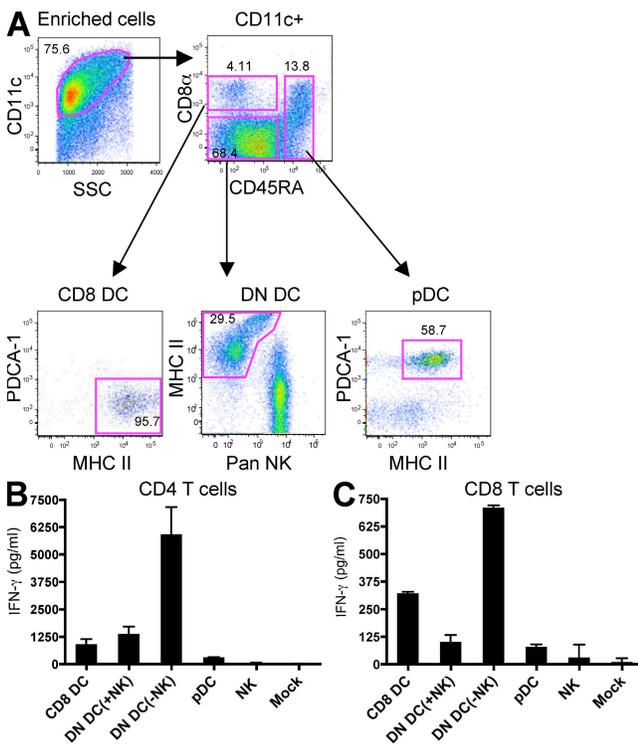


Figure 2. NK cell contamination impairs DN DC presentation of viral antigens to CD4 and CD8 T cells. (A) FACS sorting strategy of DC subsets according to previously described methods. CD11c⁺ DCs were enriched by negative selection of T cells, B cells, granulocytes, and erythrocytes, and were labeled with anti-CD11c, anti-CD8, and anti-CD45RA to distinguish pDCs, CD8 DCs, and DN DCs (references 8, 10, 11). The sorted populations were analyzed for the purity of the cell types using anti-MHC II and anti-pan-NK antibodies (percentages are shown). (B and C) Using the strategy described in A, DCs from the draining lymph nodes of f.p. HSV-1-infected mice at 72 h after infection were sorted and co-cultured with HSV-specific CD4 (B) and CD8 (C) T cells for 72 h. IFN- γ was measured by ELISA. Antigen presentation by DN DC populations containing NK cell contamination (as in A) or purified DN DCs without NK cells was assessed. Similar results were obtained from three independent experiments. Data are means \pm SD. SSC, side scatter.

Mucosal antigens do not access the lymph

It is known that, within 2 h, needle f.p. inoculation of HSV-1 results in rapid drainage of the virus into the lymph node via the lymph (16), whereas ivag infection with HSV-2 does not result in direct drainage of the virus into the lymph nodes (23). To compare the fate of antigen inoculated via these routes, FITC-dextran was injected into the f.p. by needle or inoculated intravaginally by pipette. Needle injection resulted in rapid access of the antigen to the lymph and to the draining lymph node within 30 min (Fig. 4 A), and continued to accumulate for 24 h (Fig. 4 C). In contrast, ivag inoculation of the same antigen resulted in its confinement within the vaginal mucosal cavity, and no FITC-dextran was detected in the draining lymph nodes at either 30 min (Fig. 4 B) or 24 h (Fig. 4 D). Thus, these data suggested that the delay observed in antigen presentation after ivag inoculation of HSV-1 could reflect the time required for the virus to replicate within the vaginal mucosa, and for local DCs to take up the antigens from the infected keratinocytes and migrate into the draining lymph nodes.

The source of DC populations in the lymph nodes

Thus far, our data suggested that both lymph node-resident CD8 α^+ DCs and DN DCs, the latter consisting of a mixture of resident and migratory DCs, contribute to the generation of T cell responses after HSV-1 infection. To examine the contributions of the tissue-migrant versus lymph node-resident DCs in T cell priming, peripheral DCs were labeled with FITC prepared in acetone/dibutyl phthalate in the skin (31) or FITC dissolved in DMSO in the vaginal cavity, and their migration into the draining lymph nodes was assessed. At 72 h, as previously reported (32), both the epithelial cell adhesion molecule (EpcAM)⁺ Langerhans cell (33) and the dermal DC (EpcAM⁻ CD11b^{hi}) populations contained a significant fraction of FITC⁺ cells from the skin (Fig. 5 A). Similarly, both Langerhans cells as well as submucosal DCs were labeled with FITC in the vagina-draining lymph nodes (Fig. 5 B). Further, we found that migrant DCs could be identified by the high expression of CCR7. First, Langerhans cells and dermal-derived DCs (FITC⁺) were CCR7^{hi} (Fig. 5, A and B). Second, CCR7^{hi} DCs did not proliferate in response to HSV-1 infection in the lymph node (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>), consistent with the previous description of the migrant DCs (34). Moreover, CCR7^{lo} DCs did not contain FITC⁺ cells after skin and vagina labeling (Fig. 5, A and B). The high expression of CCR7 by migratory DCs is consistent with the absolute requirement of CCR7 for tissue-derived DCs, but not the blood-derived DCs, to enter cutaneous lymph nodes (35).

Of note, within the non-Langerhans cell DC population, CD8 α^+ DCs also contained a substantial fraction of FITC⁺ cells, which were all CD11b^{hi} (Fig. 5 C). In the periphery, DCs do not express the CD8 α molecule in the vaginal or skin tissues (Fig. S7, available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>). To distinguish

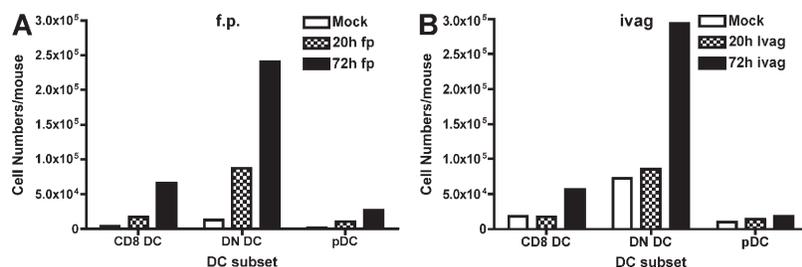


Figure 3. Relative numbers of DC subsets in the skin and vagina draining lymph nodes. Total mean numbers of DCs belonging to the three subsets in the draining lymph nodes of mice ($n = 5$ per group) infected with HSV-1 via the f.p. (popliteal; A) or the ivag (iliac and inguinal; B) route at 0, 20, and 72 h after infection were assessed. These results are representative of three similar experiments.

whether the $CD8\alpha^+ CD11b^{hi}$ cells represent lymph node-resident DCs that have picked up FITC from migrant DCs, or migrant DCs that have acquired $CD8\alpha$ expression, mice were treated with pertussis toxin (PTX) to block G protein-coupled migration (35). The emergence of the $CD8\alpha^+ FITC^+$ cells was blocked by the PTX treatment (Fig. 5 D). However, PTX treatment had no effect on the number of the lymph node-resident $CD8\alpha^+ FITC^-$ DCs (Fig. 5 D). Further, the time course of the appearance of skin-emigrant DCs and the appearance of $CD8\alpha^+ CD11b^{hi}$ cells in cutaneous lymph nodes was found to be concordant (unpublished data). To determine the possibility that the migrant DCs represent monocyte-derived TNF/inducible NO synthase (iNOS)-producing DCs (TIP DCs), their expression of TNF- α and iNOS, two characteristic functional markers of TIP DCs (36), was examined. Although a modest expression of TNF- α was detected in all DC subsets, this was not elevated in the newly migrating DCs (Fig. S8). In addition, iNOS expression was not detectable in any of the DCs in the lymph nodes, ruling out the possibility that the migrant

DCs represent TIP DCs. However, it is possible that the migrant DCs could represent monocyte-derived DCs (37) without the typical feature of the TIP DCs found in *Listeria*-infected mice. Collectively, these data indicated that $CD8\alpha^+$ DCs in the inflamed lymph nodes consist of two types: $CD8\alpha^+ CCR7^{hi} CD11b^{hi}$ cells representing tissue-emigrant DCs and $CD8\alpha^+ CCR7^{lo} CD11b^-$ cells representing lymph node-resident DCs.

Predominant role of migrant DCs in viral antigen presentation after mucosal but not epicutaneous HSV-1 infection

In the next set of experiments, we examined the capacity of the $CD8\alpha^+$ and $CD8\alpha^-$ migrant and resident DC populations in presenting the viral peptide to CD4 and CD8 T cells. To track tissue-migrant DCs, we used a previously described method to track tissue-migrant DCs after epicutaneous infection (4) and adopted the procedure for ivag infection. We were unable to use this procedure to track migrant DCs after needle injection because the needle-injected material bypassed the FITC-labeled skin tissue (unpublished data). 4 h after

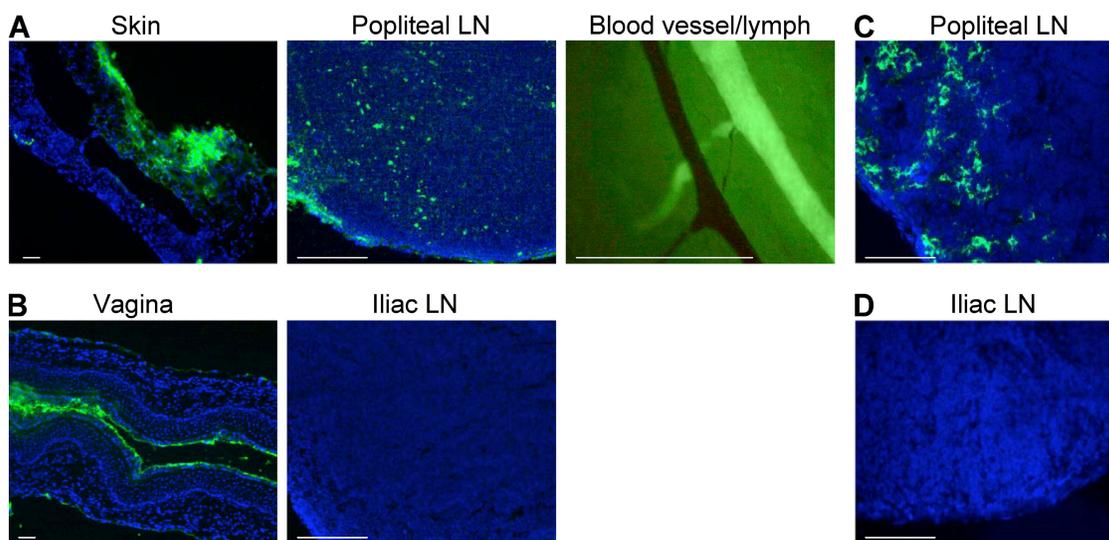


Figure 4. Mucosally applied antigens do not directly access the lymph node. Mice were injected with FITC-dextran (100 μ g per mouse) in the f.p. by needle (A and C) or into the vaginal cavity with a pipette (B and D). The applied antigen was tracked at the site of inoculation, in the lymph draining site of inoculation, and within the draining lymph nodes 30 min (A and B) or 24 h (C and D) later. These figures are representative of three similar experiments. Bars, 100 μ m.

FITC labeling of the skin or vagina (in the absence of irritant), mice were infected with HSV-1 epicutaneously (after removal of the top skin layer) or intravaginally, and the draining lymph node DCs were sorted into FITC⁺ versus FITC⁻ DC subsets. After epidermal HSV-1 infection, lymph node-resident DCs were found to be a more dominant APC population for both CD4 and CD8 T cells (Fig. S9 A, available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>), albeit at much

lower responses compared with ivag infection (Fig. S9 B). In contrast, the FITC⁺ CD8 α ⁻ DC population was found to be the most efficient APCs for both CD4 and CD8 T cells after ivag HSV-1 infection (Fig. S9 B).

To further dissect the role of migrant versus lymph node-resident DCs, we used an established definition of migrant and lymph node-resident DCs based on the level of CD205 and EpCAM expression. Both cutaneous and vagina-draining lymph

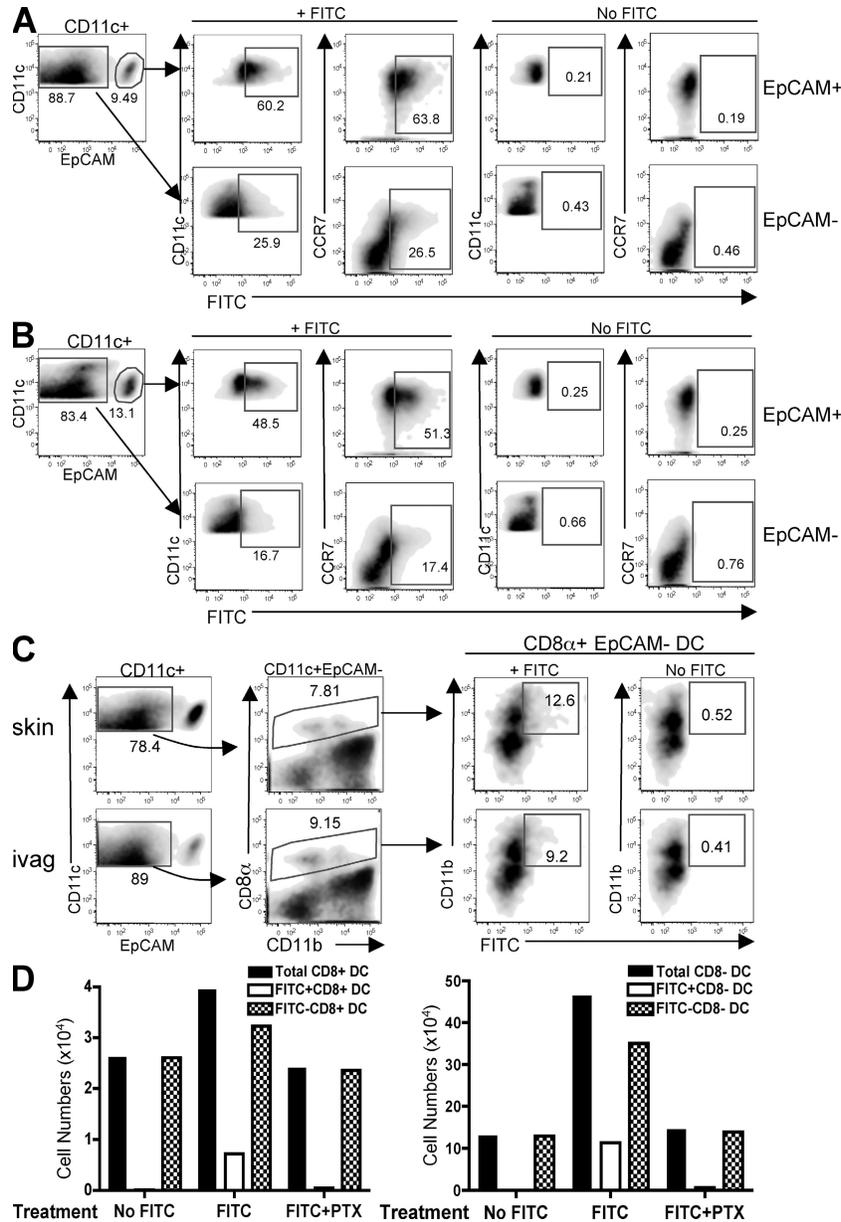


Figure 5. Characterization of the migrant versus lymph node-resident DC populations in the draining lymph nodes. Mice were painted on the flank skin with 1% FITC solution in acetone (A) or inoculated vaginally with 1% FITC in DMSO (B). At 72 h, draining lymph nodes (popliteal, A; inguinal and iliac, B) were collected, and NK cell-depleted CD11c⁺ DCs were analyzed by FACS. FITC labeling profiles for EpCAM⁺ Langerhans cells or EpCAM⁻ non-Langerhans cell DCs are depicted (percentages are shown). (C) CD11c⁺ CD8 α ⁺ EpCAM⁻ DCs were analyzed by FACS 72 h after FITC painting of the skin and FITC inoculation ivag (percentages are shown). (D) Mice were painted on the flank skin with 1% FITC solution in acetone. One group of mice received PTX injection at the site of FITC painting 18 h earlier. At 72 h, draining lymph nodes were collected, and DCs were analyzed by FACS for FITC incorporation. These figures are representative of three similar experiments.

nodes contain MHC II^{hi}, CD11c⁺, CD8 α ^{lo}, CD11b⁺, CD205^{int} (14, 38–41) EpCAM⁻ dermal/submucosal DCs (23, 33), and MHC II^{hi}, CD11c⁺, CD8 α ^{int}, CD11b⁺, CD205^{hi} (14, 38–41) EpCAM⁺ Langerhans cells (23, 33). To determine the role of migrant (Langerhans cells and submucosal DCs) versus lymph node–resident DN DCs, lymph node cells were first enriched for DCs by depleting T cells, B cells, NK cells, and erythrocytes, and CD8 α ^{hi} B220⁺ cells (Fig. 6 A). This allowed us to sort migrant submucosal DCs (CD205^{int} EpCAM⁻), Langerhans cells (CD205^{hi} EpCAM⁺), and lymph node–resident DN DCs (CD205^{lo}; Fig. 6 A) from the draining lymph nodes of ivag HSV-1–infected mice; we determined that viral antigens were presented predominantly by the submucosal CD205^{int} DCs and, to a lesser extent, by lymph node–resident CD205^{lo} DN DCs (Fig. 6 B). Langerhans cells, as confirmed by the langerin staining (Fig. 6 C), failed to present antigen to either CD8 or CD4 T cells, consistent with previous reports (11, 23). Collec-

tively, these results indicated that although both migrant and lymph node–resident DCs present viral antigens to CD4 and CD8 T cells, the predominant APCs for CD4 and CD8 T cells are the migrant submucosal DCs after mucosal infection.

Migrant DCs make contact with HSV-specific T cells after mucosal but not skin HSV-1 infection

Thus far, our data have demonstrated that the migrant DC subsets preferentially present viral antigens to CD4 and CD8 T cells after mucosal HSV-1 infection *ex vivo*. To provide *in vivo* evidence for the capacity of tissue DCs to present antigens to T cells after mucosal versus skin infection, we have analyzed DC–T cell interactions by two-photon microscopy. To track tissue DCs into the lymph nodes, we labeled skin and vaginal DCs using FITC in DMSO, as described in Fig. S9. 4 h later, HSV-1 was applied to the skin or to the vaginal cavity, and 48 h after infection, draining lymph nodes were excised and fixed immediately. DC–T cell contact was analyzed by

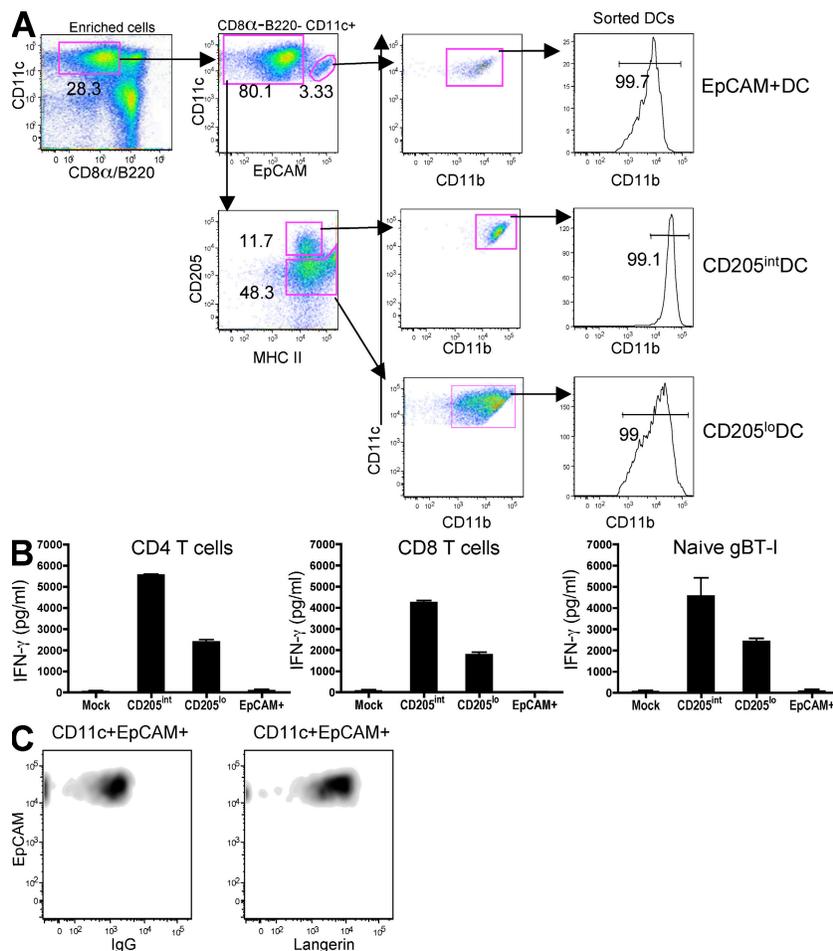


Figure 6. Migrant submucosal DCs present viral antigen to both CD4 and CD8 T cells after vaginal HSV-1 infection. (A) 72 h after ivag HSV-1 infection or mock infection, draining lymph node NK cell–depleted CD8 α ⁻ B220⁻ CD11c⁺ DCs were sorted into Langerhans cell (EpCAM⁺), submucosal DC (EpCAM⁻ CD205^{int}), and lymph node–resident DN DC (CD205^{lo}) populations. The purity of the sorted populations is indicated in the rightmost columns (percentages are shown). (B) HSV-1–specific CD4 and CD8 T cells were co-cultured with the sorted DC populations from A in the absence of exogenously added antigens. IFN- γ secretion from T cells was measured after 72 h of culture by ELISA. These data are representative of three similar experiments. Data are means \pm SD. (C) EpCAM⁻–sorted DCs (as in A, top row) were stained intracellularly with antilangerin or with an isotype control.

two-photon microscopy using Volocity software after generating volume renderings of image stacks (see Materials and methods). Enumeration of the DC–T cell conjugate in the respective lymph nodes revealed that higher frequencies of T cells make contact with migrant DCs after mucosal than epicutaneous infection (Fig. S10, available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>). Thus, these data indicated that migrant DCs interact with virus-specific CD8 T cells more frequently upon mucosal HSV-1 infection compared with skin infection, and support the notion that migrant DCs are directly involved in the priming of cognate T cells after mucosal viral infection in vivo.

DISCUSSION

In this study, we examined the contributions of DC populations in priming CTL and Th1 responses to HSV-1 infection after needle/epidermal and mucosal inoculations. Our results demonstrated that needle injection of the virus resulted in rapid acquisition of antigens by DCs in the lymph node, presumably by uptake of the lymph-borne virus in situ (27). In contrast, after mucosal infection with HSV-1, antigen-presenting DCs were only found in the draining lymph nodes after 72 h of infection, consistent with the requirement for migrant DCs to carry viral antigens to the draining lymph nodes. Examination of the DC subsets' abilities to prime T cell responses revealed that although both CD8 α^+ and CD8 α^- DCs presented viral antigens to both CD8 and CD4 T cells, the predominant APCs were the migrant CD8 α^- submucosal DCs after mucosal infection and lymph node–resident CD8 α^+ and DN DCs after cutaneous infection. These conclusions were also supported by our in vivo analyses of the interaction between the migratory DCs with HSV-specific CD8 T cells by two-photon microscopy. Migratory DCs were found in contact with cognate T cells more frequently after vaginal HSV infection than epicutaneous infection.

In contrast to previous studies (8, 10, 11), our results demonstrated the role for CD8 α^- DN DCs as APCs to CTLs after HSV-1 infection. A plausible explanation for the differences is the manner in which the DN DCs were sorted. As shown in Fig. 2, NK cell contamination interfered with the function of DN DCs. The mechanism by which NK cells inhibit the ability of DCs to stimulate CD8 T cells is unknown. Second, our data demonstrated the lack of antigen presentation by pDCs. A study has demonstrated the role of pDCs in CD4 T cell priming in the cutaneous lymph nodes using diphtheria toxin–mediated depletion of conventional DCs (cDCs) (42). Thus, in the absence of cDCs, pDCs might have a compensatory role in presenting antigens to T cells. However, our data demonstrated that in an intact mouse, pDCs do not normally participate in the priming of T cells. These data are consistent with previous reports demonstrating the role of pDCs as helpers of antigen-presenting cDCs (43) but not as direct presenters of antigens to T cells in the draining lymph nodes (44, 45). Third, we demonstrated the role of CD8 α^+ DCs in antigen presentation to CD4 T cells. In our previous study, we did not observe the ability of CD8 α^+ DCs to stim-

ulate CD4 T cell activation after HSV-2 ivag infection (23). However, we showed in this paper that the majority of this activity comes from the migrant CD8 α^+ DCs (Fig. S9 B). Thus, the overall conclusion that migrant submucosal DCs contribute significantly to CD4 T cell priming after vaginal infection remains valid for both HSV-2 and HSV-1.

Our observation that migrant DCs directly present peptides on MHC I to CD8 T cells is not without precedence. Skin-migrant DCs have been shown to present skin-derived antigen to CD8 T cells to induce activation (46) or tolerance (47). A non-CD8 α^+ DC population, particularly the dermal DC subset, in presenting antigen to both CD8 and CD4 T cells was demonstrated after f.p. injection of influenza virus (45). By tracking skin-migrant DCs with FITC, He et al. demonstrated that the migrant DCs directly presented lentiviral-encoded antigen to CD8 T cells in the draining lymph nodes (26). In this study, the authors attributed the ability of the migrant DCs to present lentiviral antigen to the nonlytic nature of the lentivirus. It is thought that a lytic virus such as HSV-1 renders directly infected DCs incapable of participating in CTL priming (26, 48–51). Therefore, our data showing that migrant submucosal DCs present HSV-1 antigens in the draining lymph nodes likely reflect antigen presentation by DCs that are not directly infected by the virus, but rather by those that acquired the viral antigens from the infected keratinocytes at the site of infection. This hypothesis is supported by the fact that there was no evidence of infected DCs that migrated to the draining lymph node after vaginal HSV-2 infection (23). Thus, extending on the previous finding with a nonlytic virus (26), the present study demonstrates that noninfected migrant submucosal DCs directly prime T cell responses after natural mucosal infection with HSV-1 by 72 h after infection.

It has long been thought that CD8 α^+ DCs exclusively arise from blood-derived precursors. Although it is true that the majority of the CD8 α^+ DCs in the spleen derive from a blood precursor and not by conversion of mature CD8 α^- DCs into CD8 α^+ DCs (52), the expression of the CD8 α molecule on tissue-derived DCs in the peripheral lymph node remains less clear. The Langerhans cells have been reported to up-regulate CD8 α expression upon maturation (53, 54). In other species such as the rat, cow, or sheep, lymph DCs include signal-regulatory protein α -negative and α -positive populations, which correspond to the CD8 α^+ and CD8 α^- DC populations in the mouse, respectively (55). More recently, skin-migrant DCs that secrete IL-12 p40 upon cutaneous LPS or *L. monocytogenes* injection were shown to express CD8 α (56). These cells are not present under a steady-state condition in the cutaneous lymph nodes, and appear to require inflammation-induced migration from the skin. Our data demonstrated that the migrant DCs clearly contained a CD8 α^+ population that coexpressed CD11b. By sorting migrant versus lymph node–resident DC populations after ivag HSV-1 infection, we found that the T cell priming activity was detected mainly in the CD8 α^- FITC $^+$ migrant DCs.

Our data demonstrated that nonmigrant lymph node–resident DCs also present viral antigen after mucosal infections.

Because viral antigens do not access the lymph node directly after mucosal infection, antigen might have been transferred from the migrant DCs (4). It is also known that cell-associated antigens are transferred to endogenous CD8 α^+ DCs in the spleen (18). In the case of cell-associated antigens, cutaneous inoculation of BM DCs loaded with nondegradable beads were found to transfer beads to endogenous lymph node DCs almost completely within 1 wk (34). Unlike these examples, because antigens associated with pathogens are likely to be degraded within 20 h to peptides within the DCs (57), and because processed peptides are not ideal substrates for cross-presentation (58), during infection, only degradation-resistant antigens may be transferred to lymph node-resident DCs for cross-presentation. Further studies are needed to unveil the nature of antigen transfer in vivo. To this end, an elegant recent study showed cooperation between lymph node-resident DCs and migrant DCs in CD4 T cell priming (59). Allenspach et al. demonstrated that lymph node-resident DCs are required to initiate activation and trapping of cognate T cells, whereas migratory DCs are required to induce proliferation. Our data are consistent with their observation in that both lymph node-resident and migrant DCs presented viral antigens regardless of the route of infection. Our observation of migrant DCs being the dominant APCs to present antigens after mucosal infection may be explained by the extent of antigenic availability in the peripheral tissue, with highly abundant antigens being presented more readily by the migrant DCs (3). Our data are consistent with this notion, because compared with the localized dermal abrasion-based HSV-1 infection, ivag HSV-1 inoculation results in the infection of the entire vaginal epithelial layer, producing very high titers of virus in the vaginal mucosa, resulting in a much more robust T cell priming compared with epicutaneous infection (Fig. S9 B). This could explain why the lymph node-resident DCs were the dominant APCs for CD8 T cells after dermal abrasion (Fig. S9 A) (4) compared with the case of mucosal infection in which the viral antigens are presented by the migrant DCs.

In conclusion, this study demonstrated the involvement of multiple DC types in T cell priming. The redundancy found in this system may reflect the necessity to ensure robust antigen presentation after viral infection. It may also reflect the requirement of these different DC subsets to perform distinct functions within the lymph node to optimally stimulate T cells (59). It is known that migrant DCs and lymph node-resident DCs differentially interact with conduits (27), endothelial cells, and T cells in distinct locations within the lymph node (60). In addition, migrant DCs are directly stimulated by innate recognition of pathogens in the periphery, whereas the lymph node DCs rely on the information carried by migrant DCs. The precise role of these DC subsets in fine tuning T cell responses remains to be resolved by future studies. The results of this study have significant implications in the strategies to design vaccines against pathogens and for immunotherapeutic approaches to treat cancer. For mucosal vaccines, our data support the strategy of steering the local DC populations to prime the type of effector responses needed in the draining lymph nodes.

MATERIALS AND METHODS

Animals. 6–8-wk-old female C57BL/6 mice were obtained from the National Cancer Institute. gBT-I transgenic mice, which express a TCR (V α 2V β 8.1) specific for the immunodominant epitope of HSV gB protein (gB₄₉₈₋₅₀₅) (61), was a gift of C. Jones and F. Carbone (University of Melbourne, Victoria, Australia), and W. Heath (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). All procedures used in this study complied with federal guidelines and institutional policies, and were approved by the Yale Animal Care and Use Committee.

Virus and infection. The HSV-1 KOS strain was provided by D. Knipe (Harvard Medical School, Boston, MA). HSV-1 was propagated and titered by a plaque assay on Vero cells. Vaginal virus infection was performed with 10⁶ PFU HSV-1, as previously described (23). For submucosal vaginal viral injection by needle, mice were anesthetized and injected with 10⁶ PFU HSV-1 in a 20- μ l volume into the submucosal tissue of the vaginal cavity by a 27G^{5/8} needle (BD). For subcutaneous infection, mice were injected with 10⁶ PFU HSV-1 in a 20- μ l volume into the hind f.p. by a 27G^{5/8} needle. For epicutaneous infection, mice were anesthetized, and the backs of mice were shaved with a hair clipper and depilated by hair remover lotion (Nair). According to a previously established method (62), small abrasions of \sim 5-mm² areas were made to remove only the top skin layer using a handheld motorized pedicure/manicure instrument (HoMedics, Inc.). No damage to the microvasculature or dermis was observed. 10⁶ PFU HSV-1 was applied on the abraded skin in a 10- μ l volume per site.

DC isolation and sorting. The draining lymph nodes were removed from mice infected at the time periods indicated in the figures, and were cut into small fragments using razor blades and digested in 2 mg/ml collagenase D (Roche) and 30 μ g/ml DNase I (Roche) at 37°C for 30 min. The cells were resuspended in HBSS containing 5% FBS and 5 mM EDTA and were incubated at 37°C for 5 min. Single cells were prepared by a 70- μ m cell strainer (BD) and were incubated for 15 min at 4°C with a mixture of rat anti-CD3 (17A2; BD), anti-Thy1 (G7; BD), anti-CD19 (1D3; BD), and antierythrocyte (TER119; BD). Cells were incubated with goat anti-rat IgG-coated Dynabeads (Invitrogen) under continuous shaking for 20 min at 4°C. Bead-absorbed cells were removed using a Dynal magnet (Invitrogen). Nondepleted cells were stained with anti-pDC antigen 1 (PDCA-1)-FITC (Miltenyi Biotec), anti-B220-PE (RA3-6B2; eBioscience), anti-CD8 α -PerCP (53-6.7; BD), anti-CD11c-allophycocyanin (HL3; BD), anti-pan-NK-biotin (DX5; eBioscience)/streptavidin-Pacific blue, and anti-MHC II (I-A/I-E; M5/114.15.2; eBioscience) antibodies conjugated by a SAIVI Alexa Fluor 680 antibody labeling kit (Invitrogen) after incubating for 10 min at 4°C in the presence of anti-CD16/32 (2.4G2; BD) antibody to block Fc receptors. Stained cells were sorted by FACSaria and analyzed with an LSR II (BD). The six-color sorting strategy allowed us to define CD8 α^+ DCs as CD11c⁺/MHC II⁺/B220⁻/CD8 α^+ /PDCA-1⁻/DX5⁻ cells, pDCs as CD11c^{int}/MHC II^{int}/B220⁺/PDCA-1⁺/DX5⁻ cells, and DN DCs as CD11c⁺/MHC II⁺/B220⁻/CD8 α^- /PDCA-1⁻/DX5⁻ cells. Sorted DCs were cultured in RPMI 1640 (Invitrogen) containing 10% FBS (Invitrogen), 50 μ M β -mercaptoethanol, 100 U/ml penicillin (Invitrogen), and 100 μ g/ml streptomycin (Invitrogen) in 96-well U-bottom plates (BD). Postsort DC populations were analyzed for MHC II expression or isotype control labeling as shown in Fig. S1 B. For the experiments depicted in Fig. 2, DCs were isolated as previously described (8, 10, 11), with the exception for data provided in the third columns of panels B and C, where NK cells were specifically depleted from the DN DC gate. For the experiments presented in Fig. 6, enriched DCs depleted of B cells, T cells, NK cells, and erythrocytes by Dynal beads were stained with anti-MHC II-FITC, anti-CD205-PE (Cedarlane Laboratories), anti-CD8 α -PerCP, anti-B220-PerCP (BD), anti-CD11c-allophycocyanin, anti-CD11b (M1/70; eBioscience)-allophycocyanin-Cy7, and anti-EpCAM-Alexa Fluor 680. CD11c⁺ B220⁻ CD8 α^- DCs were further sorted into EpCAM⁺ (Langerhans cells), EpCAM⁻ CD205^{int} (submucosal DCs), or EpCAM⁻ CD205^{lo} (DN DCs) populations. Postsort analyses indicate >95% purity of all DC populations.

T cell activation by sorted draining lymph node DCs. Naive gBT-I CD8 T cells were isolated from single-cell suspensions of lymph nodes from the gBT-I TCR transgenic mice using the CD8 T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Anti-HSV CD4 and CD8 T cells were isolated from the inguinal and iliac lymph nodes of day 7 ivag HSV-1-infected mice using anti-CD4 or anti-CD8-microbeads (Miltenyi Biotec). 2.5×10^4 FACS-sorted DCs were incubated with 10^5 CD4 or CD8 T cells for 72 h at 37°C. IFN- γ production in supernatants was measured by ELISA.

FITC-dextran injection. 100 μ g FITC-dextran (500,000 mol wt with 90 mol of dye per dextran; Invitrogen) was subcutaneously injected into hind f.p. by needle or inoculated into the vaginal cavity by a blunt-ended micropipette tip. 30 min or 24 h later, the vagina, f.p., and popliteal and iliac lymph nodes were isolated and embedded into optimum cutting temperature media. Cryosections were labeled with DAPI to visualize the nuclei. In addition, to examine FITC-dextran in the lymphatic vessels, a midline incision was made along the ventral surface of the abdominal cavity, and the skin was collected and analyzed by a fluorescence microscope.

Tracking of migrant DCs by FITC painting. Mice were anesthetized, shaved with a hair clipper, and depilated. Such areas of the skin were painted with 100 μ l of a 1% FITC solution in acetone/dibutyl phthalate (1:1 ratio; Sigma-Aldrich). The brachial lymph nodes were isolated, and single-cell suspensions were prepared as described in DC isolation and sorting. In some experiments, 0.5 μ g PTX was intradermally injected daily on the FITC-painted area to inhibit cell migration. For FITC ivag inoculation, 1% FITC solution in DMSO was inoculated into the vaginal cavity, and the draining iliac and inguinal lymph nodes were isolated at the time points indicated in the figures, and single-cell suspensions were prepared as described. Isolated lymph node cells were first enriched for DCs by depleting NK cells, T cells, B cells, and erythrocytes, and were stained with anti-CD11b-PE (M1/70), anti-CD8-PerCP, anti-CCR7-allophycocyanin (4B12; eBioscience), anti-CD11c (N418; eBioscience)-PE-Cy7, and MHC II (eBioscience)-Alexa Fluor 680 or anti-EpCAM (G8.8; BD)-Alexa Fluor 680, which were conjugated by the SAIVI Alexa Fluor 680 antibody labeling kit.

Assessment of the contributions of DC subsets per lymph node. The contributions of each DC subset in presenting viral peptides to CD8 and CD4 T cells were calculated by multiplying the ability of the sorted DC subset (25,000 DCs) to stimulate production of IFN- γ from responder T cells. For example, at 72 h after f.p. infection, the DN DC number in the popliteal lymph node was 2.5×10^5 (Fig. 3 A). It was found that 1,230 pg/ml IFN- γ was secreted from 10^5 CD8 T cells incubated with 25,000 DN DCs isolated from the popliteal lymph node at 72 h after f.p. infection (Fig. 1 H). Thus, the contribution of the DN DCs in antigen presentation to CD8 T cells at this time point was calculated as follows: 1,230 pg/ml IFN- γ /25,000 sorted DN DCs \times 2.5×10^5 DN DCs per lymph node = 12,300 pg/ml IFN- γ induced by DN DCs per lymph node.

gBT-I T cell proliferation. Naive gBT-I CD8 T cells were isolated from single-cell suspensions of lymph nodes from the gBT-I TCR transgenic mice using the CD8 T cell isolation kit according to manufacturer's instructions. Purified gBT-I CD8 T cells were labeled with 5 μ M CFSE (Invitrogen) for 10 min at 37°C. Sorted DCs were incubated with 3 μ M gB peptide for 1 h at 37°C and washed three times in PBS. gB peptide-pulsed DCs were mixed with unpulsed DCs at various ratios (the total DC number was kept constant at 10^5 DCs/well), and were co-cultured with 5×10^4 gBT-I CD8 T cells for 72 h at 37°C. Cells in the CD8 $^+$ /V α 2 $^+$ gate were analyzed as gBT-I cells after staining with anti-V α 2-PE (B20.1; BD) and anti-CD8 α -allophycocyanin (53-6.7; eBioscience), after excluding dead cells by 7-aminocincomycin D (7-AAD). CFSE dilution was analyzed by a FACSCalibur (BD). IFN- γ production from supernatants was measured by ELISA according to the manufacturer's instructions (eBioscience). In the experiments described in Fig. S2, 10^5 naive gBT-I cells were stimulated by sorted DC

populations from the draining lymph nodes of mice infected with HSV-1 at 72 h after infection. T cell proliferation was measured by 3 H incorporation after adding 0.5 μ Ci thymidine per well starting at 48 h of incubation, and radioactivity was counted at 72 h.

BrdU labeling of DCs in vivo. Mice were given 1 mg BrdU in PBS by intraperitoneal injection at 48 h after infection and were maintained with 0.8 mg/ml BrdU in drinking water for the duration of study. At 72 h after infection, iliac lymph nodes were isolated, and single cells were stained with anti-pan-NK (DX5)-PE, anti-CCR7-allophycocyanin, and anti-CD11c-PECy7 (N418). Intracellular BrdU was stained by an anti-BrdU-FITC labeling kit (BD).

Assessment of CD8 α expression on peripheral tissue DCs. Excised ear and vaginal tissue were incubated in 2 mg/ml Dispase II (Roche) for 45 min at 37°C, which allows separation of epidermal sheets and vaginal epithelium from the dermis and lamina propria, respectively. These tissues were cut into small pieces and were further digested with 0.1 mg/ml collagenase D in IMDM (Invitrogen) containing 10% FCS for 30 min at 37°C. To analyze the population in the skin dermis and the lamina propria of the vagina, these tissues were cut into small pieces and were further digested with 0.425 mg/ml collagenase D, 100 U/ml hyaluronidase (Sigma-Aldrich), and 30 μ g/ml DNase I (Sigma-Aldrich) for 60 min. These digested pieces were minced and filtered through a nylon mesh, and the resulting cells were filtered through a 70- μ m filter. Single suspensions pretreated with anti-Fc receptor antibody (eBioscience) were stained with CD11c, MHC II, and CD8 α . 7-AAD was added to the cells immediately before analysis, and 7-AAD $^+$ dead cells were excluded from analysis.

Migratory DC sorting. For FITC tracking experiments after epicutaneous infection, FITC was painted on the abraded areas of the skin, and 4 h later, 10^6 PFU HSV-1 was applied to the painted sites. For tracking experiments after ivag infection, mice were first injected ivag with a 10- μ l volume of 1% FITC solution in DMSO. 4 h later, mice were inoculated ivag with 10^6 PFU HSV-1. At 72 h after infection, brachial (for skin), and iliac and inguinal (for ivag) lymph node cells were isolated and enriched for DCs by depleting B cells, T cells, NK cells, and erythrocytes with Dynabeads, as described previously in Materials and methods, and were stained with anti-MHC II-PE, anti-CD8 α -PE-Cy7, anti-CD11c-allophycocyanin, and anti-EpCAM-Alexa Fluor 680. Stained cells were sorted by a FACSAria.

Two-photon laser scanning microscopy and three-dimensional volume rendering of excised lymph nodes. After the adoptive transfer of 5×10^5 CMRA (CellTracker Orange; Invitrogen)-labeled gBT-I T cells the day before infection, HSV-1 was epicutaneously applied to the skin or inoculated by pipette to the vaginal cavity after FITC labeling, as described in Fig. S9. A fluorescence microscope (model BX61WI; Olympus) in combination with a 20 \times 0.95NA objective (Olympus) and a multiphoton microscopy system (LaVision Biotec) was used for imaging of the excised lymph nodes. An autotunable titanium-sapphire multipass laser (Chameleon XR; Coherent) pumped by a 12W Verdi laser source was used for the excitation light source. Emitted light was collected with nondescanned detectors outfitted with the following bandpass filters: 435/90 nm (for the second harmonic emission of collagen fibers), 525/50 nm (for FITC), and 615/100 nm (for CellTracker Orange). Stacks of either 51 or 101 optical sections with a 1- μ m z spacing were acquired with the laser set at a wavelength of 820 nm. The field of view for each xy plane was either 150 μ m, at a resolution of 0.3 μ m per pixel, or 300 μ m, at a resolution of 0.59 μ m per pixel. Volocity software (PerkinElmer) was used to generate volume renderings of image stacks.

Online supplemental material. Fig. S1 depicts cell-sorting strategies to obtain pure DC populations from lymph nodes. Fig. S2 shows the proliferation of gBT-I induced by ex vivo-purified DC subsets. Fig. S3 shows the relative capacities of DC subsets in stimulating naive CD8 T cell proliferation in vitro. Fig. S4 shows antigen presentation by DC subsets after ivag

needle HSV-1 injection. Fig. S5 depicts the antigen-presenting capacity of DC subsets per lymph node. Fig. S6 shows the lack of BrdU uptake by CCR7⁺ DCs in the HSV-1 draining lymph nodes. Fig. S7 shows the lack of expression of CD8 α by local DCs in the vagina and the skin. Fig. S8 demonstrates that the migratory DCs do not express TNF- α or iNOS in the lymph node. Fig. S9 shows the relative role of migrant versus resident DCs in antigen presentation after vaginal versus epicutaneous HSV-1 infection. Fig. S10 depicts two-photon microscopic analyses of migrant DC interaction with gBT-I cells in the lymph nodes draining HSV-1 infection after vaginal versus epicutaneous delivery. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20080601/DC1>.

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